

Toxicology Excellence for Risk Assessment



TERA

*a nonprofit corporation dedicated to the
best use of toxicity data for risk values*

October 17, 1997

Addressees:

Existing Reviewers

Joe Brown, California EPA, Office of Environmental Health Hazard Assessment
Dan Caldwell, Exxon Biomedical
Dorothy Canter, U.S. EPA, Office of Solid Waste and Emergency Response
Charles Capen, Ohio State University, Department of Veterinary Biomedicine
John Christopher, California EPA, Department of Toxic Substances Control
Marvin Friedman, Cytec Industries, Inc.
Greg Harvey, U.S. Air Force, Wright-Patterson Air Force Base
Annie Jarabek, U.S. EPA, National Center for Environmental Assessment
David Morry, California EPA, Office of Environmental Health Hazard Assessment
Marilyn Underwood, California Department of Health Services

New Reviewers

Kevin Crofton, U.S. EPA, Office of Research and Development
Vicki Dellarco, U.S. EPA, Office of Water
Eric Clegg, U.S. EPA, National Center for Environmental Assessment
Kevin Mayer, U.S. EPA, Region IX
MaryJane Selgrade, U.S. EPA, Office of Research and Development

Dear Reviewers:

We wish to share with you the progress we are making with the perchlorate studies. The comments which you provided on the draft protocols were incorporated into the final with the labs selected to run the studies. The 90-day study is being conducted by Springborn Laboratories, Inc. in Spencerville, Ohio, and it was started on September 9, 1997. The neurobehavioral developmental study is being conducted by Argus Research Laboratories, Inc. in Philadelphia, Pennsylvania, and it was started on September 30, 1997. Both studies are proceeding according to the schedules presented in the protocols. Enclosed are copies of the final protocols as submitted by the testing labs and a responsiveness summary which explains how each comment from reviewers was addressed. In addition, these documents have been placed on *TERA's* homepage (www.tera.org/news).

The Perchlorate Study Group and the Air Force have also started working on the next tier of studies that will be conducted to complete the database for perchlorate. By the October 24, 1997, we will be mailing draft protocols for a 2-generation reproductive study in rats, a Segment II developmental study in rabbits (with a protocol for a dose range finding study for rabbits), and a battery of

mutagenicity/genotoxicity tests that have been sent out for bid.
We will be asking for your comments on these new protocols as
we award new contracts and develop final protocols for these new studies.



As always, we appreciate your contributions to this effort. If you have any questions or additional comments, please let me know at (513) 542-7475 or by email at dourson@tera.org.

Sincerely,

Michael L. Dourson
TERA

Enclosures

cc: C. Berrey, U.S. EPA Region IX
J. Dollarhide, *TERA*
M. Girard, PSG
D. Mattie, WPAFB
D. Rogers, WPAFB
B. Pohlmann, Nevada DEP

Responsiveness Summary: Protocol for 90-day Drinking Water Toxicity Study in Rats¹

- 1. The potential for interaction of ammonium perchlorate with other chemicals such as TCE and nitrate should be investigated.**

Since the database for ammonium perchlorate lacks some critical studies, we felt that the most important goal was to improve the toxicity database for AP first. In addition, as we determine the chemical stability of AP for the 90-Day study, we will look for nitrate formation and explore AP/nitrate interactions in solution. In the interim the Armstrong Laboratory of the Air Force will work with TCE researchers and other appropriate individuals to explore options and possible actions for future studies.

- 2. The histopathology and organ weight of pituitary gland should be collected in the 90-day study.**

These analyses have been added to the protocol as shown in Section VI.M.2-3 Experimental Design on page 19.

- 3. Since sperm evaluation is included, vaginal cytology should be studied to determine if there are any female cycling irregularities.**

This analysis has been added to the 90-day study; see Section VI.K Estrous cyclicity on page 15.

- 4. Formulated dosages should be analyzed before administration and one week after administration. If any batch is stored for a longer duration, then the last batch should also be analyzed for concentration of the dosing.**

Stock solutions of ammonium perchlorate will be prepared fresh every 4 weeks; fresh drinking water solutions will be prepared on a weekly basis from the stock solution. The concentrations of ammonium perchlorate in the stock and drinking water solutions will be analyzed on Weeks 1, 3, 5, 8, and 12 of dosing. See Section IV.D, Method and Frequency of Preparation and Section IV.E, Analysis of Dosing Preparations and Attachment 2, page 26.

- 5. Daily cage side clinical observations should be required.**

The protocol requires that animals be checked for general health/morbidity twice daily and that daily cage-side clinical

¹ Comments are in bold print. Responses follow immediately after each comment. In addition, 2 tables have been prepared and are appended to this summary. These tables give the history of the development of the RfD and the studies suggested by a peer review of the TERA RfD in March of 1997.

observations be performed. See Section VI.E, Clinical Signs, page 13.

- 6. At 14 day sacrifice, only organ weights and thyroid histopathology need to be conducted instead of complete histopathology.**

At the 14 day sacrifice, organ weights and histopathology of the liver, lung, kidney, and thyroid/parathyroid will be collected from all animals in all dose groups. Histopathology from tissues listed in Section VI.M.1 will be collected from control and high dose animals. We decided to proceed with a more complete histopathological evaluation in order to ensure that we have collected all data that might affect the risk assessment.

- 7. The recovery groups should be 10, 1, 0.05 mg/Kg BW and control.**

Groups 1, 3, 5, and 6 which receive target doses of 0, 0.05, 1.0, and 10.0 mg/kg/day, respectively, have been designated as recovery groups. See Section VI.A, Study Group Design, page 11.

- 8. The protocol should specify that the 90-day study will be performed in accordance with Good Laboratory Practices (GLP) guidelines/regulations.**

The protocol does specify that the study will be conducted in accordance with U.S. EPA Good Laboratory Practices. See Section I. Purpose, page 1.

- 9. Regarding the histopathology, all listed tissues of all animals in the second highest dose group (1.0 mg/kg/day) should be examined microscopically, as well as all tissues of high dose and control rats. The reason for this is to be able to establish a valid NOAEL should there be no pathologic findings at the high dose (10 mg/kg/day). The section on histopathology should also indicate that thyroids of all treated and control animals will be examined histopathologically, even though it is so stated elsewhere in the protocol.**

The section on histopathology (VI.M.3, page 19) does specify that the thyroids from all animals will be examined histopathologically. Conducting histopathology of all tissues in the second highest group is not required by guidelines and the cost became too high to incorporate this comment.

- 10. No mention is made in the draft protocol of who will be performing the histopathologic examinations. The protocol should contain a requirement that the contracting laboratory have a certified pathologist on staff or in a consultancy role to oversee production of the slides and to read them. Moreover, there is no mention of any quality assurance/quality control process for verifying the diagnoses of the initial pathologist who reads the slides. This needs to be addressed in the final protocol.**

The protocol specifies that histological processing will be performed by Histo Techniques, Powell, Ohio. Microscopic pathology will be performed by a Board Certified Veterinary Pathologist with experience in rodent pathology. See Section VI.M.3, page 19. Pathologists routinely double check each other's initial diagnosis and will do so in this study.

- 11. The protocol needs to be more specific as to the chemical analyses that will be performed on the ammonium perchlorate solutions to determine the stability of these solutions. Since there is some concern among peer reviewers that the ammonium ion may be converted to the nitrate ion, levels of nitrate ion in the solutions should also be analyzed.**

See response to comment #1 above. The chemical analyses are being conducted by the Armstrong Laboratory of the Air Force, who prepared a separate protocol for the analytical work. This protocol will be provided to interested parties. Armstrong Lab has been routinely analyzing for nitrate and has not seen any nitrate formation in any solutions. The stability study of ammonium perchlorate was conducted by Armstrong Lab for 60 days (6 times longer than minimum for average drinking water study) and is nearing its termination.

- 12. The protocol does not include the collection of urine from the rats.**

Urine parameters are not standard endpoints on 90-day studies especially one which has no reason to suspect the kidney as a target organ so there is no plan to include urine collection for the 90-day protocol.

- 13. The dominant lethal study is not a primary test of heritable genetic damage, but rather used in a secondary or tertiary tier of testing. However, there is no database for genetic changes so this test will be part of a battery that will include in vitro and in vivo tests. If the fertility of the males is affected by treatment for 90 days with ammonium perchlorate, then the dominant lethal assay may be of very low or no value. Sperm morphology/vaginal cytology evaluation should (SMVCE) be included at the end of the study (both for rats sacrificed after 90 days of exposure and for rats sacrificed after a subsequent 30-day recovery period). Using fewer females than suggested is not justified.**

We agree with these comments regarding the inclusion of a dominant lethal component to the 90-day study. The dominant lethal component was deleted from the study. Sperm analysis and vaginal cytology have been added to the protocol. Semen will be analyzed for total sperm count, sperm concentration, sperm motility, and sperm morphology. See Section VI.M.4, page 19. Vaginal cytology will be analyzed as discussed in #3 above. In addition, bone marrow smears will be made for micronucleus evaluation. See Section VI.M.1 Gross Necropsy, page 16.

- 14. The protocol should specify that identity, purity and stability analyses will be performed for the contracting laboratory on the ammonium perchlorate supplied by Aldrich Chemical Co. The stability of the bulk chemical should be monitored during the 90-day study to ensure that no degradation of the bulk chemical occurs.**

The protocol indicates that the Sponsor will perform all evaluations of chemical identity, purity, strength, and stability. See Section IV.A.8, page 5. The Armstrong Laboratory of the Air Force prepared separate protocols for the analytical chemistry which will be provided to interested parties.

- 15. The protocol should mention quarantining the animals for a specific period prior to commencing the 90-day study as well as the quality control procedures employed and endpoints tested (parasites?).**

The protocol indicates that the animals will be allowed to acclimate for a minimum of 10 days. During this time, the animals will be examined twice daily for general health and morbidity; any animals exhibiting abnormal signs will not be used in the study. See Section V.F, page 10.

- 16. The mechanism for individual animal identification should be specified in the protocol.**

The protocol states that after randomization, the animals will be given unique identification numbers using metal ear tags. In addition, color coded cage cards will be used to identify the study, animal and group numbers, and sex. See Section V.D., page 9.

- 17. The protocol should specify that animal cages are to be rotated every two weeks.**

Rotating cages is not required because it is not standard procedure for a 90-day study. It is required for lifetime studies.

- 18. The diet should be specified, as well as the fact that it will be available *ad libitum*. The frequency of changing the diet preparation for the cages should also be specified. The protocol should be modified to require analysis of the chow for potential contaminants, including the nitrate ion.**

The protocol specifies that the diet will be PMI certified rodent Chow #5002 from Purina Mills. This diet will be provided *ad libitum* through out the study. The protocol indicates that the feed is analyzed for environmental contaminants by the supplier and results of the analyses are maintained by the testing laboratory. See Section V.E.3, page 9. Diet preparation will be changed when the testing laboratory changes cages and measures weekly food consumption.

- 19. The protocol should indicate the frequency with which the water bottles will be changed. The protocol should specify the frequency with which a routine analysis of the deionized water will be performed.**

The protocol states that fresh water/test solution will be provided at a minimum of once weekly during the study. See Section V.E.4, page 10. The testing laboratory analyzes the deionized water periodically for environmental contaminants. In addition, the Armstrong Laboratory of the Air Force routinely analyzes the deionized water that is used to make up the dosing solutions when the ammonium perchlorate solutions are analyzed.

- 20. The protocol should specify that all animals will be observed twice daily both for viability and clinical signs. The protocol should also state that moribund animals will be sacrificed.**

The protocol states that animals will be observed twice daily; see Section VI.E, page 13. The protocol states that moribund animals will be sacrificed; see Section VI.L, page 16.

- 21. A minimum of 15 (20 is better) rats/group/time point are required to get good thyroid hormone data.**

There were differences among reviewers regarding the appropriate study design for a mechanistic study compared with a study conducted for regulatory purposes. Additional discussions with reviewers indicated that that while 15-20 rats are better for a mechanistic study, using 10 rats/sex/group will provide sufficient data for regulatory purposes.

22. The following conditions should be specified in the protocol to optimize the thyroid hormone data:

- **feed should not be withheld the evening prior to sacrifice**
- **every effort should be taken to maintain quiet in the animal room since excess noise, cage movement, and stress on the animals are known to influence serum levels of pituitary hormones**
- **sacrifice should be between 7-12 am with kills stratified between dose groups and controls**
- **blood should be collected under CO₂:O₂ (80:20) anesthesia from the posterior vena cava or abdominal aorta**
- **serum should be collected in a timely manner and frozen (-70°) with at least two aliquots from each rat to avoid freeze/thaw of samples**
- **all assays for a particular hormone should be done in one batch to minimize interassay variation**
- **serum hormone levels should be performed by a laboratory that does them on a regular basis with a record of proven accuracy**

The protocol specifies that the animals will not be fasted before sacrifice, that every effort will be made to minimize stress on the animals, and that blood will be collected from the inferior vena cave under CO₂ anesthesia. See Section VI.I , page 13 and VI.M.1, page 16. In addition, the protocol specifies that the blood will be collected before necropsy and that serum will be divided into three aliquots and immediately frozen. The samples will be maintained at -70° and shipped on dry ice to the Sponsor so that all hormone analyses can be performed in one batch. See Section VI.I, page 14. The Armstrong Laboratory of the Air Force is conducting the hormone analysis using a radioimmunoassay (RIA) method. The lab has previously conducted RIA analyses for thyroid hormones and uses an experienced biochemist to perform the analyses. All samples are analyzed in triplicate.

23. A more detailed protocol is needed for the procedures for obtaining thyroid weights in order to obtain the best data.

The protocol covers these concerns - the thyroids will be fixed first (within a minimum of 48 hours) and the same person will collect all of thyroid weights to ensure consistency.

24. The focus of the evaluation of perchlorate's effect on the thyroid should be primarily the 90-day study and not the reproduction study.

The 90-day study is the primary study which evaluates the effect of perchlorate on the thyroid. However, some thyroid parameters will also be measured in the neurobehavioral developmental study to ensure that animals have been dosed at levels that cause thyroid hormone alterations. Data on thyroid hormones will be useful to establish that perchlorate was given at high enough doses to have an effect on the thyroid in the absence of effects on the neurobehavioral measures.

25. The Air Force and PSG should look at talent in RTP area for assistance.

We will continue to interact with appropriate experts such as Kevin Crofton and Charles Capen and attempt to plan studies that answer the risk assessment (regulatory) issues for AP.

26. Look at iodine in the body after exposure to perchlorate.

We have explored this option and have proposed a separate iodine kinetic study as a complimentary study to a kinetic study for perchlorate that was proposed by Dr John Frazier (AL/OET). To add it to the 90-day study would not be cost effective nor allow sampling at more appropriate time points or more appropriate body fluids and tissues.

Responsiveness Summary: A Neurobehavioral Developmental Study of Ammonium Perchlorate Administered Orally in Drinking Water to Rats

- 1. It would help to follow Chernoff's assay procedure rather than the outlined developmental protocol. The Chernoff protocol examines the neonatal target organ toxicity.**

Available data on the effects of congenital deficit of T4 in neonates suggests that decreased T4 during the last trimester and in early neonatal development can profoundly affect IQ and learning ability later in life. Based on this information, both the March *ITER* peer review panel and the May protocol review panel recommended that the most important study to identify developmental effects of perchlorate would be a study that examined neurobehavioral endpoints. However, we recognize the importance of determining if perchlorate has any other developmental effects. Therefore, we are hoping to initiate a separate developmental segment II study if funding can be obtained.

- 2. In place of wire bottom cages during gestation and lactation, polycarbonate shoe boxes with non-reactive bedding material and nesting materials should be used. After successful mating, bred females should be transferred to the boxes containing sterilized bedding and nesting materials. MFO-induction has been reported in some bedding materials such as soft pine shavings.**

The protocol specifies that rats will not be housed in wire-bottom cages during the postpartum periods. Beginning no later than day 20 of gestation, female rats will be transferred to nesting boxes containing bed-o-cobs bedding. See the Section on Animal Husbandry, page 6-7.

- 3. In the neurobehavioral developmental study, the emphasis should be on the functional studies rather than on detailed neuropathology. Brain tissue should certainly be saved for possible subsequent evaluation of selected parameters. Some of the neuropathology studies proposed would yield more meaningful data if the brains were fixed by perfusion.**

As described in the protocol (Section on Behavioral and Developmental Observations, page 12-15), the primary focus of the study will be to evaluate the effects of ammonium perchlorate on neurobehavioral development in the F1 generation rats. Neurobehavioral function will be assessed using a variety of tests including evaluation of motor activity, auditory startle habituation, passive avoidance testing, and water testing. Brains from selected F1 generation pups at day 12 postpartum and from adult F1 generation rats after completion of behavioral testing will be evaluated for both brain weight measurements and neurohistological evaluation. See Attachment 2 of the protocol

4. Dosing should be continued until the end of the lactation period (postnatal day 21).

The primary purpose of this study is to evaluate the effects of fetal and early neonatal perchlorate exposure on neurobehavioral endpoints. Dosing is not continued through the end of lactation because after postnatal day 10, the pups begin drinking water from the water bottles and we don't want to confuse the issue by letting pups get dosed independently. Dosing through postnatal day 21 is more appropriate for a study which evaluates the mechanism of action of perchlorate on developmental endpoints. Such a study may be initiated in the future after the data needed for regulatory purposes has been collected.

5. Neurotox guidelines clearly require positive control data for all methods. Do the potential test labs have positive control data for developmental neurotox studies? What chemicals will be used to satisfy this requirement?

The protocol specifies that for all behavioral tests, data will be provided to demonstrate that the test system is capable of detecting changes produced by positive control substances (Testing Facility Positive Control Data). See page 13.

6. Why are the fetal and pup compartments being ignored for T3/T4/TSH measurement? There is a distinct possibility that these compartments may be more sensitive to the effects of perchlorate. I would recommend the addition of fetal and early postnatal time points for the determination of T3, T4, and TSH.

We will bleed the culled pups at day 5 followed by obtaining thyroid weights and histopath of thyroids. We are also looking at obtaining thyroids and blood again at the end of the study. A formal amendment to the protocol will be added to TERA's homepage (www.tera.org/news) as soon as it is available.

7. Overall, the methods description for the behavioral tests are too vague to allow an adequate assessment of whether these tests will be of any use. Description should include information on positive controls, whether the method has been published, how many trials will be required and how many days the testing will take.

The final protocol describes the behavioral tests in much greater detail and now contains the information requested by this comment. See the Section on Behavioral Observations, page 12-15.

Studies and Areas of Scientific Uncertainty In Reference Doses

| STUDY | Description | H | A | S | D | L | Study's Usefulness |
|--|---|---|---|---|---|---|--|
| 1. Neurobehavioral Developmental | tests nervous system of fetal, newborn and young animals | X | | | X | | tests whether young animals are more sensitive than adults; <u>may</u> reduce H and will reduce D factor |
| 2. 90-day, all other organs | tests many organs of young adult animals | | | | X | | gives sufficient information to generate an RfD; will reduce D factor |
| 3. Receptor kinetics (in vitro studies; perchlorate discharge tests) | compares how perchlorate is absorbed, metabolized, and excreted in animals & humans | X | X | | | | shows if uncertainty factors for H and A can be changed from default values of 10 |
| 4. Segment II developmental | tests for birth defects | | | | X | | will reduce D factor |
| 5. ADME - Absorption, Distribution, Metabolism and Elimination | compares how perchlorate is absorbed, metabolized, and excreted in animals and humans | X | X | | X | | shows if uncertainty factors for H and A can be changed from default values of 10; <u>may</u> affect value of D factor |
| 6. Mutagenicity/ Genotoxicity | tests for mutations and toxic effects on DNA | | | X | X | | <u>may</u> affect value of S and D factor |
| 7. Reproductive | tests for reproductive performance in adults, and for toxicity in young animals | | | | X | | will reduce D factor |
| 8. Immunotoxicity | tests for immunotoxicity in adults | | | | X | | <u>may</u> reduce value of D factor |

Uncertainty factors for developing RfDs are as follows:

H = average human to sensitive human

S = short term to long term studies

D = data base deficiencies

A = animal to man

L = LOAEL to NOAEL

History of Reference Doses (RfDs) and Areas of Scientific Uncertainty

| Item \ Group | EPA 1992 | EPA 1995 | TERA 2/97 | Peer Review 3/7/97* | New Data (ongoing)** |
|--|------------------------|------------------------|-----------------------|------------------------|-------------------------|
| Confidence in RfD | low | low | medium to low | data not sufficient | medium |
| Study: BMD/NOAEL/LOAEL (mg/kg-day) | Human NOAEL 0.14 | Human NOAEL 0.14 | Human LOAEL 1.4 | Rat BMD 0.9 | Rat BMD ? |
| Area of Uncertainty: | | | | | |
| Within Human | 10 | 10 | 3 | 10 | 10 |
| Animal to Human | 1 | 1 | 1 | 1 | 1 |
| Subchronic to chronic | 10 | 10 | 3 | 3 - 10 | 1 - 3 |
| LOAEL to NOAEL | 1 | 1 | 3 | 1 | 1 |
| Data Base | 10 | 3 | 3 | 10 | 1 - 3 |
| Total Factor | 1000 | 300 | 100 | 1000 | 100 |
| RfD (mg/kg-day) | 0.0001 | 0.0005 | 0.01 | 0.0009 | ? |

*The peer review panel thought that the data were insufficient to develop an RfD. However, when pressed several panel members stated that the use of a benchmark dose and the listed uncertainty factors might be reasonable (www.tera.org).

**Uncertainty factors in this column are reasonable estimates; they may or may not represent chosen values after the new studies are completed.

BMD = benchmark dose; NOAEL = no observed adverse effect level; LOAEL = lowest observed adverse effect level